COMPOSITION OF HONEY. VII. PROTEINS*

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SUMMARY

Crude protein preparations from a number of types of floral honey, and from stores of sugar-fed confined bees, have been examined by gel filtration, starch-gel electrophoresis and ion-exchange chromatography.

Between 4 and 7 protein components are indicated by gel electrophoresis, with differences among floral types implying the presence of plant protein components. Four components, apparently from the bees, are common to all samples.

For four honey samples, 35% – 60% of the total nitrogen content passed through the dialysis membrane; hence, the protein content cannot be determined from the nitrogen content of whole honey.

INTRODUCTION

Honey is primarily a carbohydrate material, with an average of 95 – 97% of its solids in this category. Relatively little has been published on honey proteins in general, although the enzymes have received much attention in Europe as indicators of table quality. Some years ago the colloidal constituents as a class, including proteinaceous material, were examined from a processing viewpoint (Paine, Gertler & Lothrop, 1934).

The nitrogen content of 490 samples of U.S. honey (White, Riethof, Subers & Kushnir, 1962) ranged from 0·000 to 0·133%, with an average of 0·041% (S = 0·026); possibly this relatively low apparent protein content (0·25%) has been responsible for the lack of previous interest. Level of protein content as indicated by the formol titration (Tillmans & Kiesgen, 1927) or precipitation (Lund, 1909; Voerman & Bakker, 1911) has been used in Europe as a test for adulteration. Other early tests for honey adulteration involved the precipitation of honey proteins by either anti-bee serum (Thöni, 1911) or a rabbit antiserum to honey proteins (Langer, 1909). Moreau (1911) reported honey to contain albumin, globulins, proteoses and peptones; Stitz (1930) found peptones, globulins and albumins, but could not detect 'protamines, alcohol-soluble albumins, histones, albumoses and albuminoids'. More recently Helvey (1953) examined the non-dialysable constituents of buckwheat honey by sedimentation and moving-boundary electrophoresis. The colloidal material of this honey (total 0·28%) was sedimented in three fractions; the largest (50%) had a molecular weight 146 000, the next (35%) 73 000†, and the smallest (15%) 9000. By moving-

* The previous paper in this series is by White, Riethof and Kushnir (1961).

† Recalculation of the average in Helvey's paper gives about 80 000.

boundary electrophoresis three distinct components were found, in about the same proportions.

The proteins in honey might originate from the plant nectar, from the honeybee, or from pollen. Using Sephadex gel filtration, starch gel electrophoresis, and ion exchange chromatography, we have examined the colloidal materials of honey as obtained by dialysis. A sample of 'honey' produced by confined bees when fed sucrose syrup has also been studied; the honeybee origin of many of the protein fractions is thus demonstrated.

MATERIALS AND METHODS

Honey samples

All were unheated and were either purchased in 60-lb containers or obtained as small samples from producers for a recent analytical survey of U.S. honey (White et al., 1962). They had been stored at refrigerator temperature (1 week) or at −20°C (several years) since receipt. Two special samples were included, a nine-day collection of autumn honey (sample M 260, primarily goldenrod, i.e. *Solidago*) and one obtained by feeding caged bees with sucrose syrup, with special precautions to prevent admixture with honey or nectar from plants (sample M 259). The first (M 260) was stored by a normal free-flying colony of bees in freshly drawn comb which had been extracted with water to remove traces of honey. Collection was from 9th to 18th September 1961. Comb was removed (half capped) and held in a freezer until extracted by hand. For the sugar-fed sample (M 259) $1\frac{1}{2}-2$ kg of bees in a cage were starved 2 days and then fed a few litres of sugar syrup for two days to flush out any traces of residual honey from the bees. The stored syrup was removed, fresh water-extracted comb was given, and sucrose syrup (50%) was fed from 14th to 18th September. The comb was removed two days later and held at −20° until extraction. Both samples were crushed, drained, and strained at room temperature, then returned to freezing storage until used.

Preparation of concentrates

For dialysis, 25 g honey were diluted with 15 ml water containing 12 1 Merthiolate (Eli Lilly and Company brand of sodium ethylmercurithiosalicy and held at 37° for 30 min in a tightly closed dialysis tube (2·5 cm made of regenerated cellulose, to eliminate bacterial contamination. dialysis against running tap water for 16 hr, volume in the tube was red from about 75 ml as needed, by treatment of the closed tube at 4° with sodium carboxymethyl cellulose (Aquacide I, Calbiochem, Los Angeles, fornia). Traces of sugars remaining had no effect on subsequent treatment second 4-hr dialysis then removed ultraviolet-absorbing material originating f the Aquacide treatment.

*Mention of trade or company names does not imply endorsement by the Departn over others of a similar nature not named.

Gel filtration

Columns (bed 2·1 × 31 cm) were prepared of Sephadex (bead type) (Pharmacia, Uppsala, Sweden) and washed exhaustively with 0·01-M phosphate, *p*H 6·5 before use. They were stored at 4° and used at 25°, with flow rates of 10 – 15ml/hr. Fractions of 2 ml were collected.

Gel electrophoresis

Starch-gel electrophoresis was performed generally as outlined by Smithies (1955, 1959), using hydrolysed starch he recommended (Connaught Medical Research Labs., Toronto). Procedural details appear elsewhere (White & Kushnir, 1966).

Ion-exchange chromatography

DEAE-cellulose (2·5g) after decantation of fines was treated 0·5 hr with 100 ml 0·1 M NaOH, filtered, dispersed three times in 2 litres of water, and twice in 500 ml 0·01 M potassium phosphate, *p*H 8·0. A thick slurry in phosphate was placed in a column with continuous gentle suction to give a bed 0·8 × 1·8 cm which was washed several hours with the buffer. Fractions collected were 2·0 ml, with a linear gradient of KCl applied after about 25 fractions, rising from 0 to about 0·5 M at fraction 100. Between fractionations the exchanger was removed from the column and regenerated as above.

Protein determination

Protein was estimated in column effluent fractions by the Warburg and Christian optical procedure according to the equation given by Layne (1957): protein in mg/ml = $1{\cdot}55\ A_{280} - 0{\cdot}76A_{260}$. The procedure of Lowry et al. (1951), calibrated with twice-recrystallized egg albumin, was also used. Nitrogen content of some preparations was also determined by a micro-Kjeldahl procedure.

RESULTS

Protein analysis of preparations

Samples of 13 honeys (11 floral types), and one produced by sugar feeding, were dialysed and concentrated as described above. For control purposes the original honey, and the dialysed material after concentration, were assayed for protein by one or more of the three procedures.

The second column of Table 1 shows the 'protein' content of the original honey sample as nitrogen × 6·25. The remaining data give analyses of the dialysed material, all expressed as mg protein per g of the original honey represented in the preparation. For five of the samples protein content was calculated from the nitrogen contents of the dialysed and concentrated material (final column).

Starch-gel electrophoresis

Results of nigrosine staining of honey protein bands separated by starch-gel electrophoresis are shown in Fig. 1, which includes nine floral types of honey and

TABLE 1. Protein content of preparations from honey

Floral type		*Apparent protein* *				*N loss on dialysis* %
		Before dialysis $N \times 6{\cdot}25$	*After dialysis & concentration*			
			Optical	*Lowry*	$N \times 6{\cdot}25$	
Goldenrod-aster *Solidago-Aster*	(HS 33)	5·75		5·75		
	(HS 35)	10·6	13·7	11·1		
	(HS 37)	10·2	12·1	6·88	6·43	37
Clover *Trifolium*	(HS 36)	2·16	2·4	0·96	0·91	58
Cotton *Gossypium*	(HS 38)	4·21	6·8	2·05	1·65	61
Spanish needle *Bidens*	(430)†	3·68	8·9			
Basswood *Tilia*	(M 264)		4·9	2·45		
White clover *Trifolium repens*	(236)	2·25	3·84			
Tupelo *Nyssa*	(467)	2·93	5·50			
Sugar-feeding	(M 259)	1·37	1·49	0·84	0·84	38
Goldenrod	(M 260)		7·19	3·17	2·68	
Lespedeza	(359)	3·94		1·56		
Wingstem-*Lespedeza* *Actinomeris alternifolia-Lespedeza*	(490)	3·19		1·53		
Tulip tree *Liriodendron tulipifera*	(454)	6·12	10·6	2·80		

* Mg per gram original honey.
† Numbers without letters represent samples described elsewhere (White *et al.*, 1962).

the sample of 'honey' produced by sugar-feeding. The broken line connects components of corresponding mobility in each set. Samples *a*, *f*, *j* and *n* different preparations from the same honey sample; they were included to pro relative identification of the bands of other samples. Different distance migration in the sets are due to applied voltages, times, and age of starch.

Gel filtration

A protein concentrate from a honey sample, prepared as described was subjected to gel filtration on columns of Sephadex G-25, G-50, G-10 G-200 in 0·01 M phosphate at *p*H 6·5. Apparent protein in the 2-ml e fractions was determined optically. Best fractionation resulted from the filtration, which was used for the work described here.

TABLE 2. Sample preparation for gel filtration

Floral source		Amount dialysed	Final volume *	Protein in conc.†	Protein on column
		g	ml		mg
Tupelo	(467)	10	2·21	5·50	12·4
Basswood	(M 264)	20	5·02	4·87	9·7
Spanish needle	(430)	10	1·58	3·84	12·0
Goldenrod-aster	(HS 35)	20	9·0	13·7	15·3
,, ,,	(HS 37)	40	4·17	12·0	14·4
Clover	(HS 36)	40	3·60	2·4	13·3
Cotton	(HS 38)	40	4·91	5·9	23·6
Sugar-feeding	(M 259)	40	1·80	1·24	6·9

* volume after dialysis and concentration
† mg per g honey

Table 2 gives details of sample preparation for eight samples and shows the amount of material applied to the column, usually in 0·25 – 0·5 ml. In Fig. 2 and 3 are the effluent diagrams of these samples, representing seven honeys and one from sugar feeding. Protein in the eluates was determined optically (solid line) and by the Lowry method (broken line). To determine which method better reflects actual protein concentration, a gel filtration was done and the fractions combined (Fig. 4*a*). These fractions were analysed by Lowry, optical, and micro-Kjeldahl methods, with results seen in Table 3.

TABLE 3. Protein content of fractions from gel filtration of honey protein preparation *

Fraction *	Apparent protein content (mg)		
	Optical	Lowry	N × 6·25
A	11·9	3·15	2·66
B	24·8	22·0	21·0
C	21·6	15·0	12·7
D	14·2	4·6	4·3

* See Fig. 4*a*

Ion-exchange chromatography

A sample (0·25 ml) of one of the preparations of Table 2 (HS 37) was subjected to chromatography on DEAE-cellulose with a KCl gradient. Part of the 'protein' (optical) passed through the column, and part was retained and

TABLE 4. Ion-exchange chromatography of honey protein concentrate

	Distribution of 'protein' *	
	Adsorbed mg	Not adsorbed mg
First adsorption	13·0 (85%)	3·4 (15%)
Second adsorption:		
Adsorbed material from first run	2·6 (90%)	0·28 (10%)
Unadsorbed material from first run	0·68 (82%)	0·15 (18%)

* analysed optically

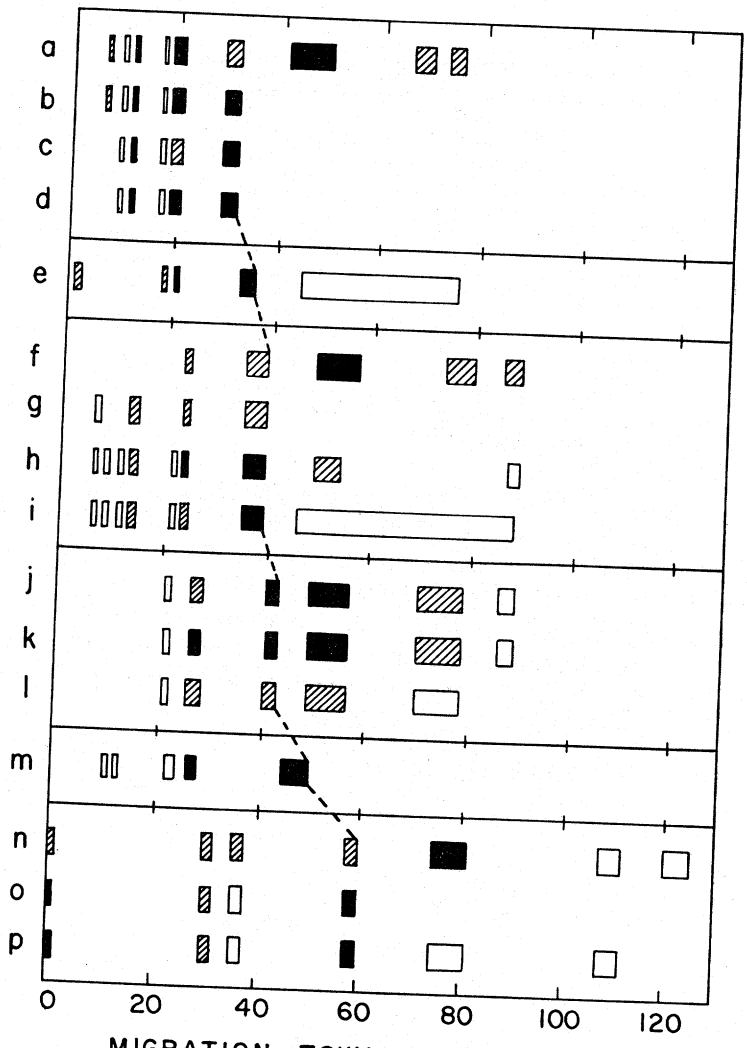


FIG. 1. Diagram of starch-gel electrophoresis (2–4°) of honey proteins in borate (*p*H 8·9), stained with nigrosine. Samples enclosed in each rectangle run on same gel.

At 2·82 v/cm, 17 hr
1*a*: Goldenrod-Aster (HS 37)
1*b*: Tupelo (467)
1*c*: Spanish needle (430)
1*d*: Basswood (M 264)

At 2·84 v/cm, 16 hr:
1*e*: Tulip tree (454)

At 3·04 v/cm, 16 hr:
1*f*: Goldenrod-Aster (HS 37)
1*g*: Stores from sugar-feeding (M 259)
1*h*: Goldenrod (M 260)
1*i*: Cotton (HS 38)

At 3·52 v/cm, 16 hr:
1*j*: Goldenrod-Aster (HS 33)
1*k*: Goldenrod-Aster (HS 35)
1*l*: Goldenrod-Aster (HS 37)

At 3·54 v/cm, 16 hr:
1*m*: Clover (HS 36)

At 3·58 v/cm, 17 hr:
1*n*: Goldenrod-Aster (HS 37)
1*o*: Wingstem-*Lespedeza* (490)
1*p*: *Lespedeza* (350)

Samples in *a, f, j, n* are different preparations from a single honey sample, included reference. Intensity of dye indicated by 3 levels of shading.

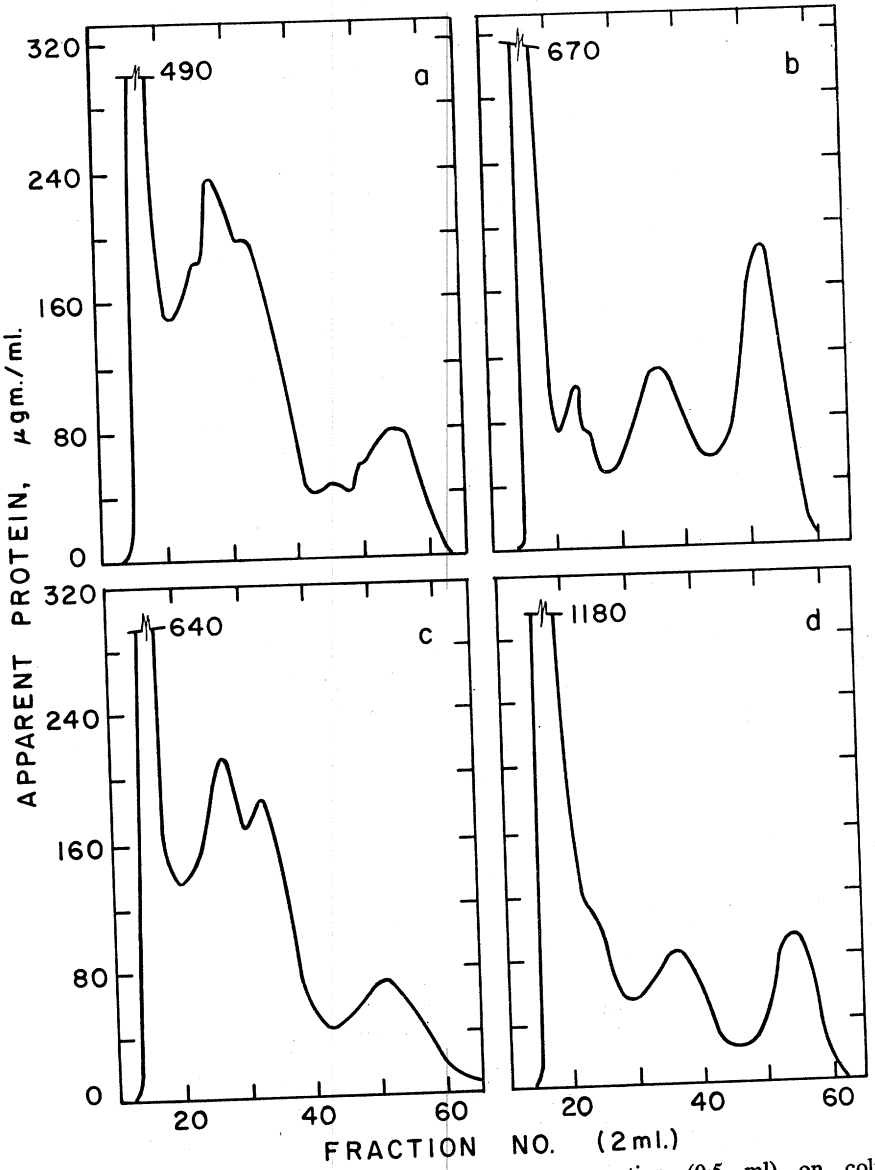


FIG. 2. Filtration of concentrated dialysed honey preparation (0·5 ml) on columns (2·1 × 31 mm) of Sephadex G–200. Solid lines: apparent protein determined by optical method

2*a*: Goldenrod-Aster (HS 35)

2*b*: Basswood (M 264)

2*c*: Goldenrod-Aster (HS 37)

2*d*: Tupelo (467)

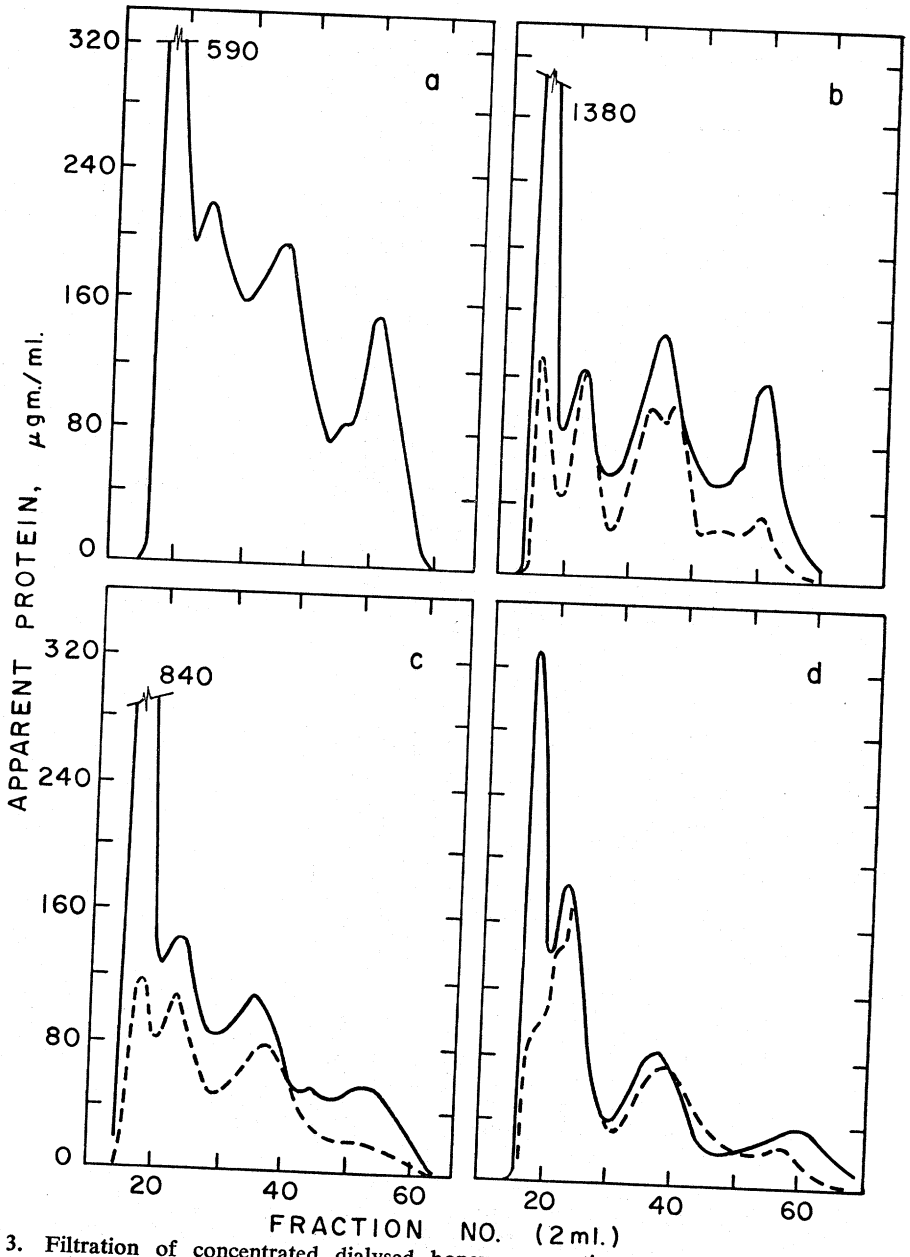


FIG. 3. Filtration of concentrated dialysed honey preparations (0·5 ml) on columns of Sephadex G-200. Solid lines: apparent protein by optical method. Broken lines: apparent protein by Lowry method.

3a: Spanish needle (430)

3b: Cotton (HS 38)

3c: Clover (HS 36)

3d: Stores from sugar-fed bees (M 259)

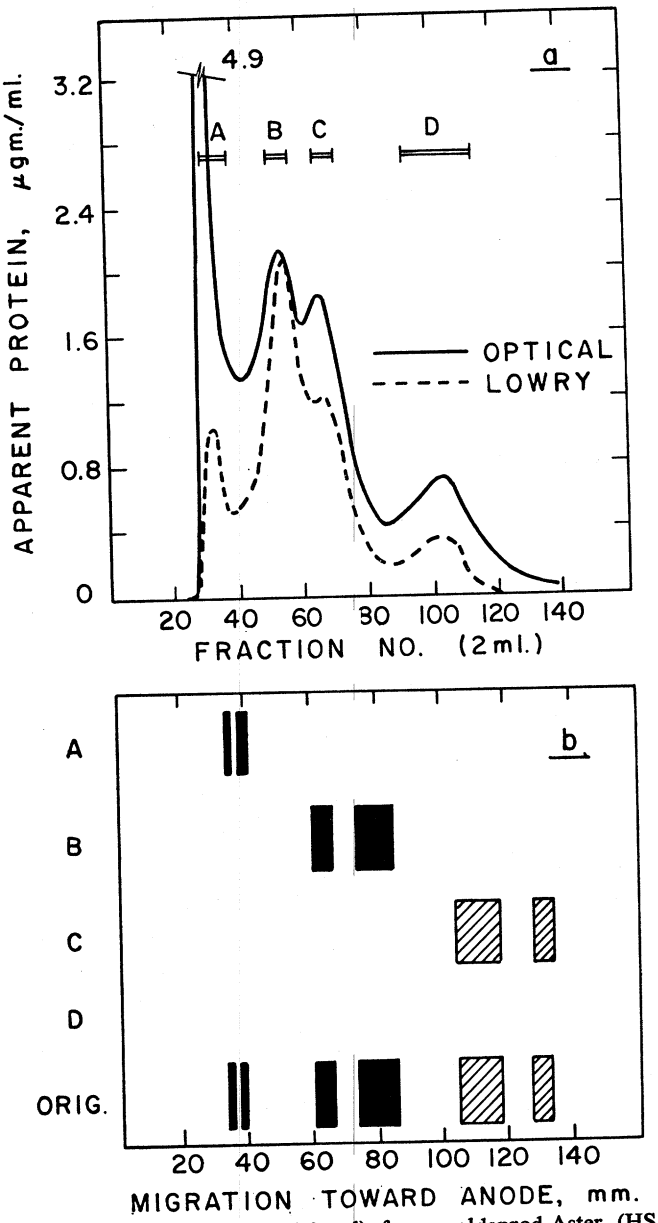


FIG. 4*a*: Gel filtration of preparation (1·0 ml) from goldenrod-Aster (HS 37) honey, on column (bed volume 2·1 × 60 cm) of Sephadex G-200. Fractions combined as shown. 4*b*: Fractions A-D from Fig. 4*a* subjected to starch-gel electrophoresis. Pattern at bottom from original material as applied to gel column.

partially fractionated by the gradient. These two fractions (retained and not retained) were dialysed, concentrated with Aquacide I, and applied to fresh columns of the same size. Again each was divided into two portions—adsorbed and unadsorbed—in about the same proportion as originally (Table 4). The amounts of material applied were much less than the loading capacity of the column. A possible interchange between two forms would explain this behaviour. Ion-exchange chromatographic analysis of several samples is shown in Fig. 5, 6 and 7.

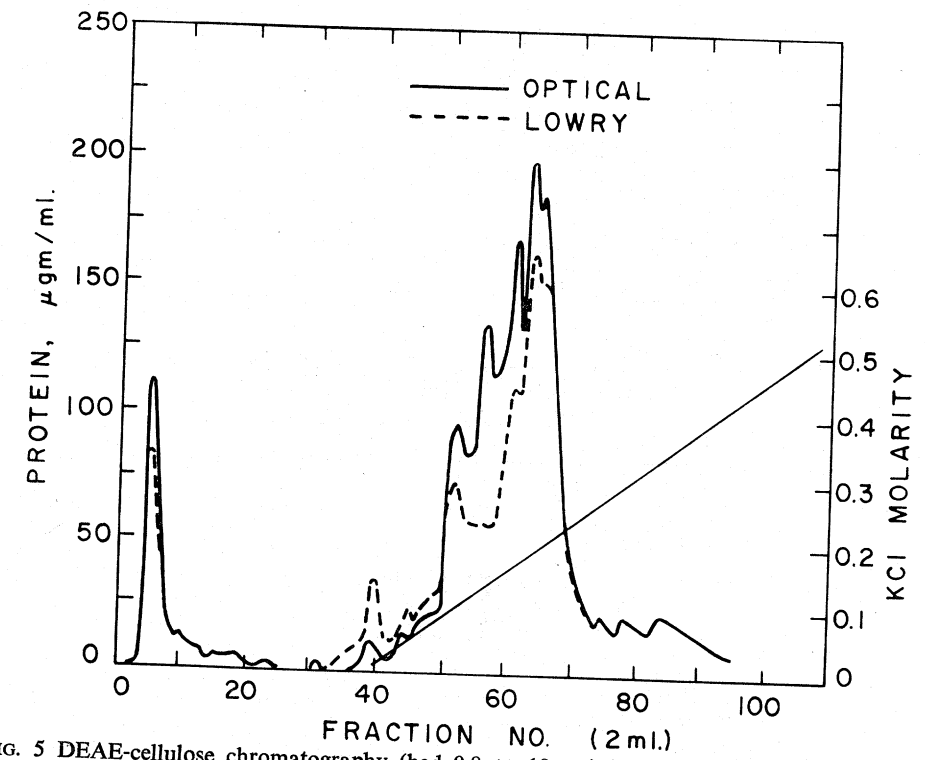
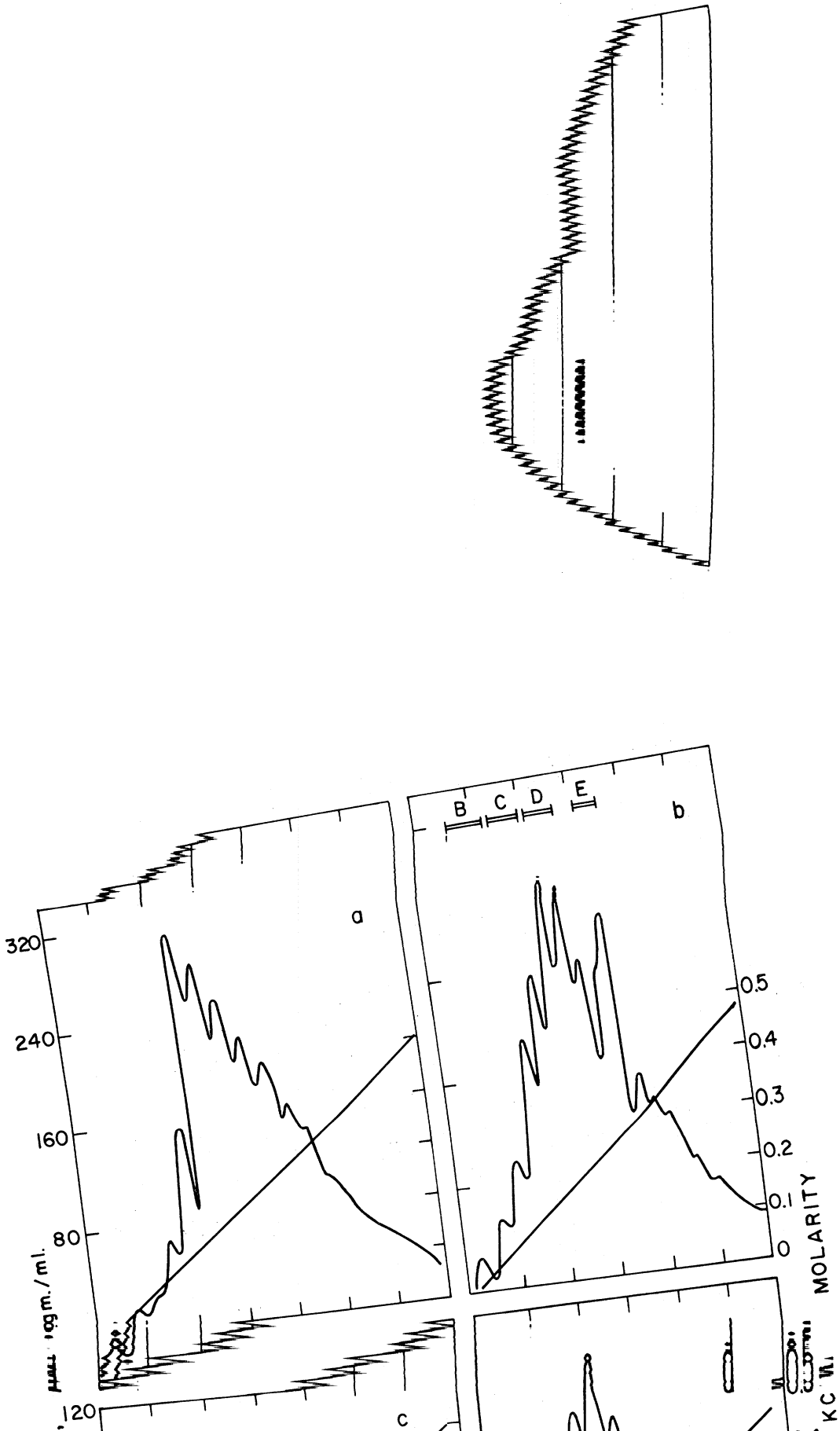


FIG. 5 DEAE-cellulose chromatography (bed 0·8 × 18 cm) in 0·01-M phosphate (*p*H 8·0 of 0·5 ml dialysed protein concentrate from clover honey (HS 36). Solid line: apparent protein by optical method. Broken line: by Lowry method (scales left). Straight line: gradient of salt concentration (scale right).

DISCUSSION

In general, the Warburg-Christian optical method for protein analysis results much higher than the other two. For the five samples whose Lowry Kjeldahl values are both given (Table 1), these are in reasonable agreem considering the fact that the Lowry procedure was calibrated with egg albu and a factor of 6·25 was arbitrarily used with the nitrogen conversion.

Comparison of columns 2 and 5 shows (for the four samples analysed large loss of nitrogen compounds in dialysis, with half passing through membrane for two of the samples. The practice of estimating protein content


B
C
D
E
a
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0.5
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0.2
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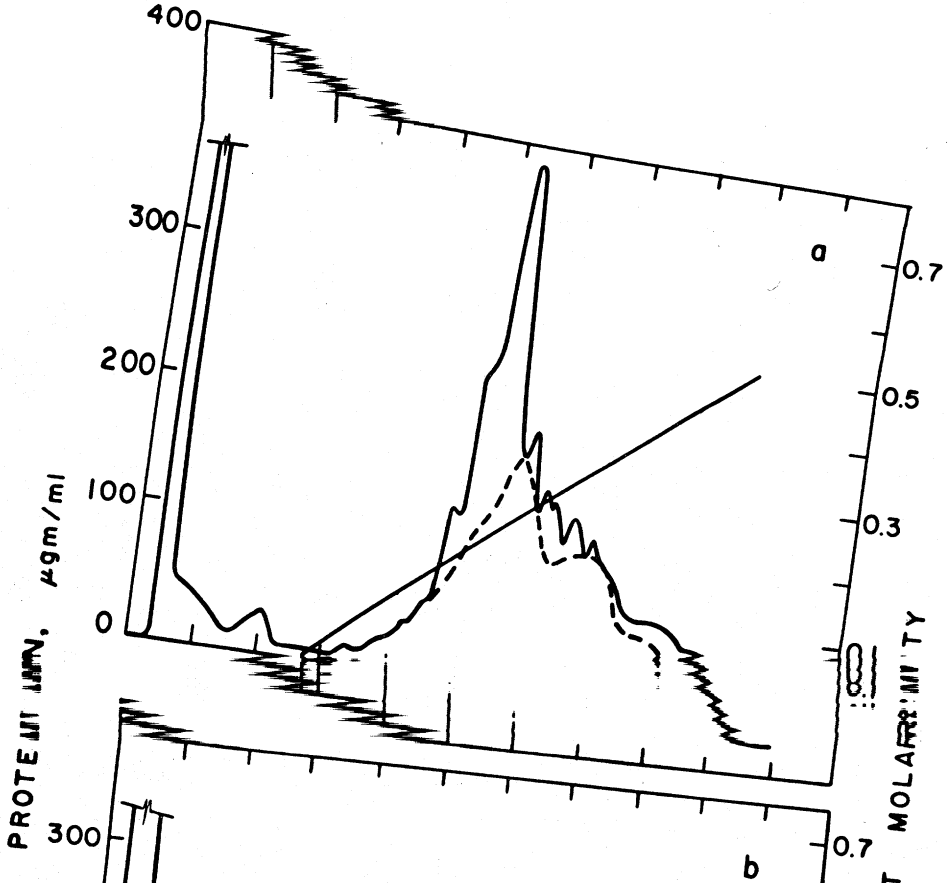
MOLARITY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PROTEIN, µgm/ml
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b
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MOLARITY

honey from its nitrogen content is thus seen to give erroneously high values, presumably because it records considerable amounts of non-protein nitrogen.

Two general types of electrophoretic pattern may be seen in Fig. 1. Nearly all samples had more or less of diffuse dyed background (which is not indicated in the diagrams), extending from the origin to the most mobile edge. Nearly all samples, if examined at sufficiently high concentration, show the pattern of two doublets and a single heavier band which is seen in Fig. 1*b, c, d, m.* In addition, all four preparations from goldenrod-aster honey (so-called fall-flower honey) showed three further rapidly-migrating bands: one, very marked, just beyond the principal band in all samples, and two more diffuse bands at the limit of migration. Patterns from tulip-tree (Fig. 1*e*) and cotton (Fig. 1*i*) had a broad diffuse area of staining in this region, without band structure.

The sample prepared from stores taken from sugar-fed bee (Fig. 1*g*) shows only single bands in the areas of the paired bands seen in the honey protein samples. This material was quite low in protein content, all of which must have originated in the bees. It is not known if the use of higher concentrations would have revealed pairing of these bands, since at higher concentrations protein precipitated from the preparation.

All the gel filtration effluent diagrams in Fig. 2 and 3 show a relatively large amount of excluded material by optical measurement. Both elution positions and relative amounts of the various retarded fractions differ among the samples shown in Fig. 2 and 3. By comparison with the pattern in Fig. 3*d* (from sugar-fed bees), it appears that plant components contribute to several samples (Fig. 2*a, c, d*). The pattern in 3*c*, from a low-protein clover honey, is similar to that in 3*d*. The patterns in 2*a* and 2*c* are from samples of goldenrod-aster honey which have a higher protein content, and also a different gel-electrophoretic pattern from the other samples examined.

When protein is determined in the eluant fractions by the Lowry method (Fig. 3*b, c, d,* 4*a*), a more meaningful curve results. It is seen that the excluded fraction is equal to or less than the others in amount; much of the ultraviolet-absorbing material in this fraction is thus neither protein or carbohydrate (non-reactive with anthrone-sulphuric acid). Any dispersed beeswax in the honey would appear here. On the basis of the ratio of the peaks at 18 and 22 ml in Fig. 3*d* (no plant components) it appears that each of the other samples (Fig. 3*b, c,* 4*a*) contains appreciable protein from the plant. It is evident in Table 3 that, for filtrate fractions, results by the Lowry method are in reasonable agreement with those obtained from nitrogen content; this was also true for the preparations in general. Differences are of the order expected when the Lowry procedure, calibrated against one protein, is applied to others. The optical method is seen to give variably high results when applied to these eluates: much non-protein, non-nucleotide, ultraviolet-absorbing material is present. A graph of carbohydrate content (by anthrone) in the effluent showed no similarity to a graph of the difference between protein content measured by the two analytical methods. The gel-filtrate fractions, combined into the four portions shown in Fig. 4*a*, were reduced in volume by Aquacide, then redialysed to remove ultraviolet-absorbing material imparted by this treatment, and subjected to starch-gel electrophoresis.

Fig. 4*b* show the migration of the constituents; the last sample on the diagram is the original preparation as applied to the Sephadex column in Fig. 4*a*. This sample is HS 37, goldenrod-aster, whose electrophoretic pattern is shown also in Fig. 1*k*. The size-selecting aspects of both separation methods are evident from examination of Figs. 4*a* and 4*b*.

Approximate molecular weights were calculated, by the procedure outlined by Leach and O'Shea (1965), for several peaks in the effluent curves analysed by the Lowry method. For HS 37, goldenrod-aster (Fig. 4*a*), the values are higher than 400 000, 98 000, 40 000, 3400; for the clover sample (HS 36, Fig. 3*c*) higher than 400 000, 260 000, 45 000. The two major peaks for the sample from sugar-feeding (M 259, Fig. 3*d*) gave 240 000 and 40 000, the shape of the first peak to emerge indicating some material of higher weight.

In Fig. 5 the complete elution diagram is shown for a preparation from clover honey, and Fig. 6 shows fractionation on DEAE-cellulose of the adsorbed part of preparations from three other honeys and the sugar-fed sample. The differences in the fine structure of the eluted band are fairly reproducible for samples of the same honey type. It should be noted that the elution curves shown by broken lines (representing protein by the Lowry procedure) show a relatively simpler structure than that of the solid lines (optical determination). Other ultraviolet-

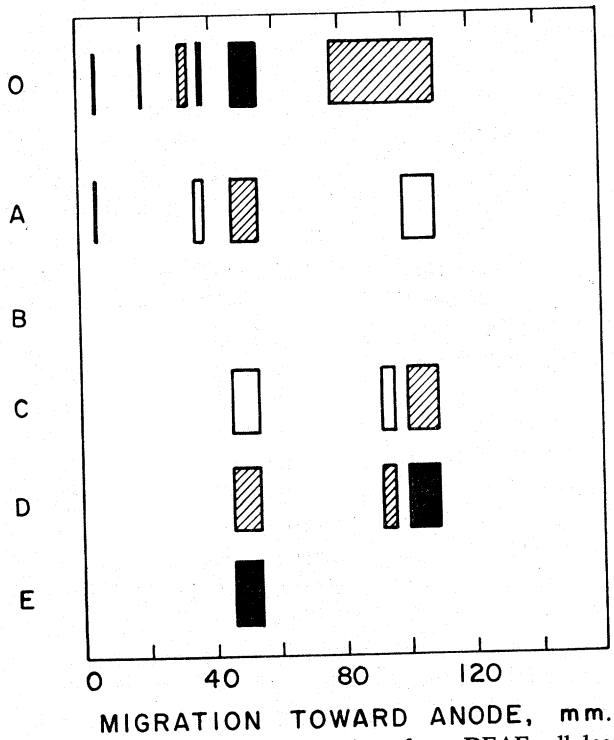

FIG. 8. Starch-gel electrophoresis of pooled fractions from DEAE-cellulose chromatography of cotton honey protein concentrate (cf. Fig. 6*b*). 3·68 v/cm, 16 hr borate (*p*H 8·9). Original concentrate (*O*) shown at top. *A* is material not absorbed on column. Other fractions as shown in Fig. 6*b*. Apparent protein content, by optical method, as applied to gel: *O*, 5·6 mg/ml; *A*, 3·70; *B*, 1·03; *C*, 1·43; *D*, 1·05; *E*, 1·31.

absorbing non-dialysable components are evidently being fractionated; in some of the diagrams they account for a third or more of the adsorbed material.

Fig. 6*c*, *d* and 7*a* show that most of the non-protein material (where the largest difference between the two curves is seen) is in the portion eluted at lower salt concentrations. If the fractionation is carried out in a sodium borate buffer with NaCl gradient, instead of in phosphate, much of the non-protein material appears earlier and is better separated from the protein, as shown in Fig. 7*b*. Analysis by anthrone shows the essentially non-sugar character of this material.

The four fractions *B*-*E* shown in the chromatogram of Fig. 6*b*, plus the unadsorbed fraction *A*, were dialysed, reduced in volume with Aquacide I, redialysed and subjected to starch-gel electrophoresis. 'Protein' contents (optical) are shown for each in the legend for Fig. 8. In Fig. 8 the material requiring the highest salt concentration for elution is seen to be the most prominent electrophoretic band, but not the most mobile. The material not retained by the ion-exchange column (fraction *A*) contains four of the bands of the original material. This, together with the apparent equilibration between adsorbed material and unadsorbed material, may imply the existence of two forms of these components, one in which binding groups are available to the column and one in which they are not.

CONCLUSIONS

The number of constituents in the protein fraction of honey varies among the several floral types examined from 4 to at least 7; 4 of these components appear to originate in the bee. Molecular weights of two of the bee-imparted proteins were indicated by gel filtration to be around 40 000 and 240 000; other protein materials from the plant showed values of about 98 000 and above 400 000. The preparation contained several high-molecular non-protein materials, shown by ultraviolet analysis; these were fractionated by ion-exchange chromatography, in addition to the protein components noted above.

The portion of nitrogen-containing material of sufficiently low molecular size to pass through the dialysis membrane varies from sample to sample; in the few examined here 35% – 60% did so. Obviously the protein content of honey cannot be determined from the nitrogen content of whole honey.

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Bee World, the official organ of the Association, which provides beekeeper and scientist with news and articles on recent developments.

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